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Ectopic F_0F_1 ATP synthase contains both nuclear and mitochondrially-encoded subunits

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Abstract Over the past few years, several reports have described the presence of F_0F_1 ATP synthase subunits at the surface of hepatocytes, where the hydrolytic activity of F_1 sector faces outside and triggers HDL endocytosis. An intriguing question is whether the *ectopic* enzyme has same subunit composition and molecular mass as that of the mitochondrial ATP synthase. Also due to the polar nature of hepatocytes, the enzyme may be localized to a particular cell boundary. Using different methods to prepare rat liver plasma membranes, which have been subjected to digitonin extraction, *hr* CN PAGE, immunoblotting, and mass spectrometry analysis, we demonstrate the presence of ecto-F_0F_1 complexes which have a similar molecular weight to the monomeric form of the mitochondrial complexes, containing both nuclear and mitochondrially-encoded subunits. This finding makes it unlikely that the enzyme assembles on the plasma membranes, but suggest it to be transported whole after being assembled in mitochondria by still unknown pathways. Moreover, the plasma membrane preparation enriched in basolateral proteins contains much higher amounts of complete and active F_0F_1 complexes, consistent with their specific function to modulate the HDL uptake on hepatocyte surface.

Keywords *hr* CN PAGE and LC-MS/MS · F_0F_1 ATP synthase · Plasma membrane · Basolateral and Apical membrane of hepatocytes

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Introduction

Although previously the F_0F_1 ATP synthase, the terminal enzyme of the mitochondrial oxidative phosphorylation system, was thought to be confined to the inner mitochondrial membrane, recent immunofluorescence, proteomic and functional studies including ours (Vantourout et al. 2010; Giorgio et al. 2010; Contessi et al. 2007) have demonstrated the presence of subunits of both the catalytic F_1 and the membrane-embedded F_0 sector on the cell surface of wide variety of normal and tumor cells. While the mitochondrial enzyme is primarily deputed to the aerobic synthesis of ATP (Boyer 1997), the surface enzyme has been implicated in cell signaling mediating various biological functions, such as the regulation of lipid metabolism and immune recognition of tumors (Champagne et al. 2006; Chi and Pizzo 2006), but also playing pathogenic roles in different diseases (Gorai et al. 2012; Vacirca et al. 2012; Chang et al. 2012). Due to its expression at the cell surface with the F_1 subunits facing

outwards, the enzyme has been designated ectopic or cell surface ATP synthase/ATPase. Consistent with its role in cell signaling, the ectopic enzyme seems to be mainly localized in lipid rafts (Kim et al. 2006; Bae et al. 2004), which are membrane microdomains rich in cholesterol and sphingolipids harboring various signaling molecules. In hepatocytes, the ectopic β chain is the high-affinity apo-AI receptor (Martinez et al. 2003), and is believed to trigger extracellular ATP hydrolysis and, consequently, ADP-dependent activation of P2Y13, a G protein-coupled purinergic receptor participating in HDL endocytosis (Malaval et al. 2009).

Although all these pieces of experimental evidences suggest that the ectopically-expressed ATP synthase is structurally similar to the mitochondrial F_0F_1 complex, its subunit composition and its identity with the mitochondrial enzyme have not been definitively proven. The mammalian complex has at least 15 subunits (Wittig and Schägger 2008), of which five form the F_1 sector with the stoichiometry $\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$. The F_0 part comprises a ring structure formed by eight c-subunits which is connected at the top to the central stalk, constituted by the γ, δ and ε subunits, and peripherally to the proton-conducting a subunit (Watt et al. 2010) and the lateral stalk formed by b, d, F6 and OSCP subunits. Also associated with the enzyme are 'minor subunits' e, f, g and A6L, all of which span the membrane (Baker et al. 2012). In addition, the complex can bind the inhibitor protein IF_1, which reversibly binds to F_1 and fully inhibits the hydrolytic activity (Bason et al. 2011; Harris and Das 1991).

The majority of these subunits are nuclear gene products. However, in mammals, subunits a and A6L are encoded by mitochondrial genome. This arrangement highlights the complexity of enzyme assembly, which requires accessory factors whose definition is still under investigation (Rak et al. 2011a).

Regarding the ectopic enzyme, most reports describe the presence of the α and β subunits on the cell surface by flow cytometry or confocal microscopy. In some studies, the presence of other F_1 and F_0 subunits have been demonstrated, but the mitochondrial encoded subunits have not previously been identified (Vantourout et al. 2010). This is a critical point, because their detection would make the enzyme assembly on the plasma membranes unlikely, as it is unlikely that these subunits are targeted both to the cell surface and to mitochondria.

In our previous paper we demonstrated that rat liver plasma membranes contain F_0F_1 complexes displaying similar molecular weight to the mitochondrial F_0F_1 ATP synthase, but their molecular composition was not defined due to the very low amount of enzyme obtained (Giorgio et al. 2010). In this study, we improved the isolation procedure of F_0F_1 complexes from rat liver plasma membranes and this allowed their analysis by LC-MS/MS, which established the presence of both nuclear and mitochondrially-encoded subunits. Moreover, rat liver is made up of polarized cells with distinct basolateral and canalicular membranes involved in uptake from the portal blood and in bile secretion, respectively. The results below indicate that plasma membrane preparations enriched in basolateral proteins contain much higher amounts of active F_0F_1 complex, in accordance with its postulated function in modulating HDL uptake.

Materials and methods

Mitochondria and plasma membranes isolation from rat liver

All animals were harvested and killed at the Animal House of the Department of Pharmaceutical and Pharmacological Sciences, University of Padova according to the provisions of the European Community Council Directive (n. 86/609/CEE) and of the Italian legislation (D.L.vo 116/92). Adult male Sprague Dawley rats, with a mean weight of 300–350 g, were chosen. Livers were excised, and plasma membranes and mitochondria were immediately isolated. Mitochondria were prepared by a standard method (Giorgio et al. 2009), while plasma membranes were obtained using two different protocols.

Method A: plasma membranes were isolated essentially as described by Van Amelsvoort et al. (1978). Briefly, tissue pieces were homogenized with a Teflon-glass Potter-Elvehjem homogenizer in a buffer containing 0.25 M Sucrose, 10 mM HEPES, 0.2 mM $CaCl_2$, pH 7.4 (HM1). 1 mM EDTA was added and the suspension centrifuged at 1000 g for 10 min. The supernatant was centrifuged at 25.000 g for 30 min, and microsomal and plasma membranes fractions were separated as a white fluffy pellet over tight pellet of mitochondria. Finally, the plasma membrane pellet was re-homogenized in HM1 supplemented with 1 mM EDTA and layered on a discontinuous sucrose gradient (in 10 mM Hepes pH 7.4), composed of 39 % and 20 % sucrose (mass:vol), and centrifuged at 50.000*g* for 3 h. Plasma membranes were recovered from the interface of the heavy-light sucrose solutions, diluted 3 times with HM1 and harvested at 70.000*g* for 40 min to obtain PMA. All the operations were carried out at 4 °C.

Method B: plasma membranes were isolated essentially as described by Harris et al. (2005). Briefly, tissue pieces were suspended in a buffer containing 0.25 M sucrose, 1 mM $MgCl_2$, 10 mM Tris–HCl, pH 7.4 (HM2) (approximately 4 ml/g of liver), homogenized with a Teflon-glass Potter-Elvehjem homogeniser, filtered through a nylon mesh of 750–1000 μm pore size, diluted in HM2 buffer to 5 ml/g of liver and finally centrifuged at 300 g for 5 min. 50 ml aliquots of the supernatant were re-centrifuged at 1500 g for 10 min and the pellets, containing the nuclei and plasma membranes, were re-suspended in 25 ml of HM2 buffer and mixed with 50 ml of dense sucrose solution (2 M sucrose, 10 mM Tris–

HCl, pH 7.4) to obtain a final density of 1.18 g/ml. The suspension was divided in 25 ml aliquots, each overlaid with 1.5 ml of HM2 and centrifuged at 113000 g for 1 h. Plasma membrane sheets floated upwards and were collected from the interface, diluted four times with HM2 buffer and harvested by centrifugation at 8000 g for 10 min to obtain PMB.

To separate basolateral and apical membranes, 2 mg of PMB were homogenized using 30 vigorous strokes of a tight fitting Potter-Elvehjem homogenizer in suspension buffer containing 0.25 M sucrose, 10 mM Tris–HCl, pH 7.4 (SB). The suspension was laid over discontinuous gradient of 44 %, 39 % and 34 % sucrose in SB and spun down at 196000 g for 3 h. Apical membranes were collected at the interface of suspension and 35 % sucrose and basolateral membranes were collected from the interphase of 39 % and 44 % sucrose. After dilution with 2 volumes of SB, membranes were harvested by centrifugation at 200000 g for 30 min. All the operations were carried out at 4 °C (Cefaratti et al. 2000).

Detergent extraction, gel electrophoresis, immuno-precipitation and immuno-blotting

Plasma membrane and mitochondrial proteins (0.5 mg) were pelleted by centrifugation for 20 min at 4 °C, at 100.000 g or 25.000 g, respectively. Pellets were suspended in 50 μl of a solubilization buffer containing 50 mM imidazole, 500 mM 6-aminohexanoic acid, 1 mM EDTA, pH 7 at 4 °C (Wittig et al. 2006). Digitonin (catalog number 37006 Fluka, used without recrystallization) was added to a final concentration ranging from 0.5 to 4 g/g of protein and incubated at 4 °C (for mitochondria) or 37 °C (for plasma membranes) for 20 min (Kim et al. 2004; Nielsen et al. 2005). The suspension was centrifuged at 100.000 g, 30 min at 4 °C and the supernatant was retained.

For high resolution clear native electrophoresis (*hr* CN PAGE), 20 μl of the digitonin extracts were supplemented with 2 μl loading dye (0.1 % Ponceau in 50 % glycerol) (Wittig et al. 2007). 5 μl samples were loaded for mitochondria and 20 μl for plasma membranes (due to difference in the content/activity of ATP synthase in mitochondria and plasma membranes) and separated on linear gradient of 5–11 % acrylamide gel using as cathode buffer 50 mM Tricine, 7.5 mM imidazole supplemented with 0.05 % DOC (Merck) and 0.05 % Triton X-100 (Fluka), as specified in (Wittig et al. 2007). Electrophoresis was run at 180 V constant power for 30 min and finally to 250 V for remaining 1.5 h maintaining the temperature at 4 °C to keep the enzyme in its native state. Gels were either stained for ATPase activity according to Zerbetto et al. (1997) +/− oligomycin as specified in (Wittig et al. 2007) or transferred to nitrocellulose membranes for native immunoblotting using a semidry electroblotting system (Bio-Rad). Before blotting the gels were incubated for 1 h in 25 mM Tris, 192 mM Glycine and 0.1 % SDS to impart a slight negative charge to proteins and facilitate their transfer. No SDS was incorporated in the transfer buffer.

For immunoprecipitation of the whole F_0F_1 complex, PMB digitonin extracts were treated with anti-complex V monoclonal antibody covalently linked to protein G-Agarose beads (MS501 immunocapture kit, abcam®) in a ratio of 20 μl of conjugated beads per mg of membrane protein according to the manufacturer's instructions. IP supernatants were separated by SDS-PAGE followed by immunoblotting containing SDS in transfer buffer.

The antibodies used were polyclonal (rabbit) anti-NTCP (sc-98484, Santa Cruz Biotechnology) (1:1000), anti BSEP (sc-25571, Santa Cruz Biotechnology) (1:1000) anti-Flotillin 1 (F1180, Sigma) (1:2000), anti-Tom 20 (FL 145, Santa Cruz Biotechnology), anti-TERA (PA5-17486, Santa Cruz) (1:1000), anti-VDAC (sc-98708, Santa Cruz Biotechnology), in-house polyclonal antibodies against F_1 α/β subunits and Complex II (Loro et al. 2009), monoclonal (mouse) antibodies anti-ATP5B (ab14730) (1:2000), anti-Clathrin (C 1860, Sigma) (1:1000). Blots were probed using Hrp conjugated antibodies anti-rabbit (1:10000) and anti-mouse (1:5000) (Sigma). The blots were developed using Pierce ECL Plus western blotting substrate (P180196), imaged using VERSADOC, BioRad and quantified using Quantity one, BioRad.

Protein identification by mass spectrometry

After the separation of proteins by *hr* CN PAGE, followed by in-gel ATPase staining, the bands were excised and destained by incubation overnight with 10 % acetic acid. In-gel digestion was performed as previously described (Bozzo et al. 2005). Protein digests were then resuspended in 0.1 % formic acid and analysed by LC-MS/MS. MS analysis was performed at the Proteomic Center of Padua University, VIMM and Padua University Hospital (Padua, Italy) on a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Pittsburgh, CA, USA) coupled with an on-line nano-HPLC Ultimate 3000 (Dionex, Thermo Fisher Scientific). Samples were loaded onto a 10 cm fused silica emitter with a 75 μm inner diameter that was packed in-house with Magic C18 AQ 5 μm resin (Michrom BioResources, Auburn, CA, USA). The samples were separated at a flow rate of 250 nl/min using a gradient from 3 % B to 40 % B in 39 min (solvent A: H_2O with 0.1 % formic acid; solvent B: acetonitrile with 0.1 % formic acid). The MS/MS acquisition method was based on a full-scan on the Orbitrap with a resolution of 60,000, followed by the isolation for fragmentation of the most intense ions (up to ten) in the linear ion-trap. MS/MS data were searched using the MASCOT search engine (Matrix Science Ltd., London, UK) against the *Rattus norvegicus* sequences of the Swiss-Prot database (release 2011_05, 7620 sequences). The following parameters were used in the MASCOT search: trypsin specificity; maximum number of missed cleavages 1; fixed modification, carbamidomethyl (Cys); variable modifications,

oxidation (Met); peptide mass tolerance, ± 10 ppm; fragment mass tolerance, ± 0.6 Da; protein mass, unrestricted; mass values, monoisotopic. Protein hits were required to include at least one bold red peptide match and the ions score cut-off was set to a value of 20. The False Discovery Rate (FDR) for peptides above identity threshold was calculated using the decoy feature of Mascot and it was set to a value of ≤1 %. A protein hit was designated when two unique peptides with statistically significant scores (FDR ≤1 %) were obtained. For two cases in which the identification was based on only one peptide (subunit a and ε in Table 1), the identification was validated manually based on visual inspection of the MS/MS spectra.

Results

ATP synthase extracted from rat liver plasma membranes has the same molecular mass as the mitochondrial complex

Two methods of plasma membrane isolation from rat liver were used which are based on markedly different approaches.

Table 1 List of the proteins identified by LC-MS/MS[a] in the *hr* CN PAGE bands containing the F_0F_1 complexes extracted from mitochondria. F_0F_1 bands were excised, destained with 10 % acetic acid and subjected to mass spectrometry analysis as described in Materials and methods

Sector	Subunit	Mass (Da)[b]	UniProtKB AC	Coverage (%)[c]	MS/MS peptides[d]
F_1	α	59,754	P15999	57.5	36
	β	56,354	P10719	40.6	17
	γ	30,191	P35435	45.8	14
	δ	17,595	P35434	17.3	3
	ε	5,767	P29418	15.7	1[e]
F_0	a	25,050	P05504	4.4	1[e]
	b	28,869	P19511	39.5	14
	OSCP	23,398	Q06647	45.5	9
	d	18,763	P31399	42.2	8
	ATP8/A6L	7,642	P11608	46.3	3
Carrier	ADT2	32,901	Q09073	37.2	11

[a] The identified proteins with their accession numbers and molecular masses are listed. Protein identification was performed with the MASCOT software searching LC-MS/MS data against the sequences *Rattus norvegicus* of the Swiss-Prot database

[b] Molecular mass of the UniProt sequence in the absence of molecule processing

[c] Sequence coverage calculated on the basis of the peptides sequenced by LC-MS/MS

[d] Peptides sequenced by LC-MS/MS. The peptides identified for each protein with their expectation values are listed in Table S1 of the Supplementary Material

[e] The MS/MS spectra of these peptides have statistically significant scores and were manually validated

In Method A (Van Amelsvoort et al. 1978), plasma membranes (PMA) were obtained from the subcellular fraction containing microsomes and plasma membranes by a 3 h centrifugation on a discontinuous 20/39 % sucrose gradient, as previously made in (Giorgio et al. 2010). In method B (Harris et al. 2005) plasma membranes, collapsed over the nuclei, were separated as floating membranes by isopycnic centrifugation using 2 M sucrose (PMB). Considering that the ectopic enzyme is mainly localized in lipid rafts composed of cholesterol and sphingolipids (Gorai et al. 2012; Vacirca et al. 2012), both plasma membrane preparations were treated with the mild non-ionic detergent digitonin (Nielsen et al. 2005), which forms an equimolar complex with cholesterol. Moreover, digitonin was chosen being widely used to extract F_0F_1 complexes from the inner mitochondrial membrane (Wittig et al. 2006), because it maintains the ATP synthase oligomeric forms (Giorgio et al. 2009). To isolate the ectopic F_0F_1 complexes, digitonin extracts were subjected to native electrophoresis, which is a powerful approach to separate and identify membrane complexes and supramolecular assemblies in native state (Wittig et al. 2006). Native electrophoresis was followed by in-gel ATPase activity and immunoblotting with in-house polyclonal antibodies against F_1 α/β subunits (Loro et al. 2009) to detect fragments which may be formed during PM preparations. *hr* CN PAGE was performed instead of BN PAGE (Wittig et al. 2007) because of the higher sensitivity of the in-gel ATPase activity assay. This is essential to detect the enzyme extracted from plasma membranes, whose F_0F_1 content is much less than that of mitochondria, as discussed below.

Figure 1 shows that both PMA and PMB extracts contain active F_0F_1 complexes (ecto-F_0F_1) having almost the same molecular mass as the F_0F_1 monomers extracted from mitochondria. As expected, in mitochondria this extraction procedure generated both the monomeric and dimeric form of ATP synthase (Wittig et al. 2007). Both bands were detected by immunoblotting against F_1 α/β subunits (Fig. 1b) and were inhibited by oligomycin when stained by in-gel ATPase activity, thus demonstrating they were holo-F_0F_1 complexes (Suppl Fig. 1). However, plasma membranes were treated with digitonin at a temperature of 37 °C, which favors isolation of raft proteins (Kim et al. 2004), while extraction from mitochondria was carried out at 4 °C, which is the optimum condition to obtain active complexes (monomers and dimers) from mitochondrial membranes (Wittig et al. 2007).

Figure 1 shows that both methods A and B yielded active complexes, with method B giving a higher yield of ecto-F_0F_1 complexes. By measuring the densitometric ratio between the intensities of the ATPase activity band and the F_1 α/β band, ecto-F_0F_1 from PMB and PMA displays a specific activity that was 85±5 % and 56±4 % (mean ± S.E. of three independent determinations) of that of the monomeric complexes extracted

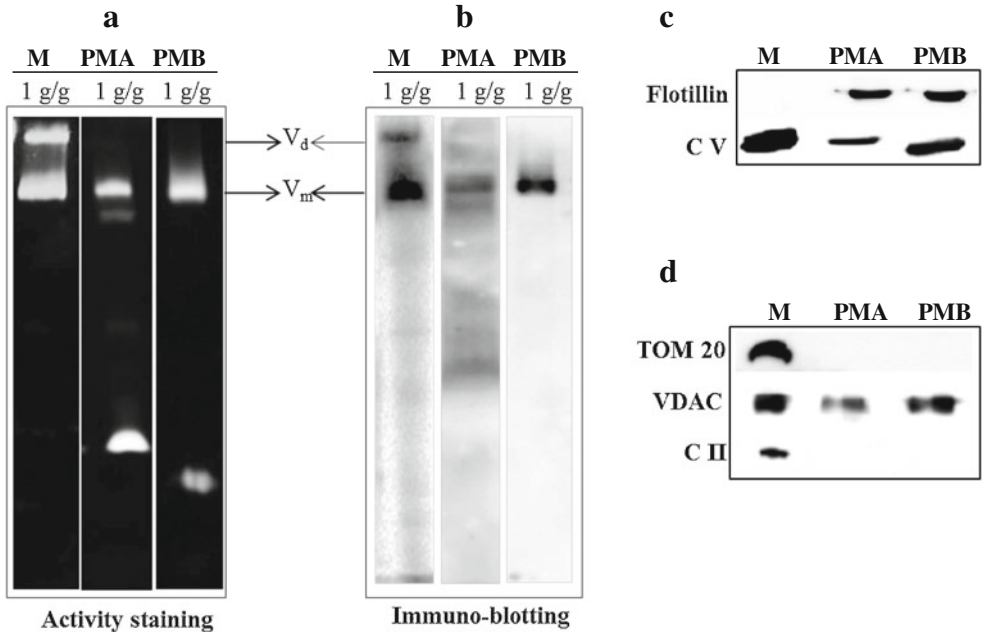

Fig. 1 *hr* CN PAGE, ATPase activity staining (**a**) and native immunoblotting (**b**), and SDS-PAGE and immunoblotting (**c** and **d**) of digitonin extracts from plasma membranes prepared by method A and B and from mitochondria. Plasma membrane and mitochondrial proteins (500 μg) were extracted using 1 g/g of digitonin (detergent/protein ratio) at 37 °C and 4 °C, respectively. Extracts were loaded (5 μl for mitochondria and 20 μl for plasma membranes) on *hr* CN PAGE and subjected to both in-gel ATPase activity staining (**a**) and immunoblotting against poly Ab α/β subunits (**b**). V_m and V_d denote monomeric and dimeric form of the mitochondrial ATP synthase. In **c** and **d** mitochondrial proteins (5 μg) and plasma membrane proteins (40 μg) were directly loaded on 12 % SDS PAGE and analyzed with monoclonal Ab against β subunit of ATP synthase (CV) or poly Ab against flotillin or anti-TOM 20 or anti-VDAC or anti-Complex II (CII)

from mitochondria taken as 100 %, respectively. In contrast to the mitochondrial extracts, neither plasma membrane extract showed ATPase dimers, while in both extracts rather intense ATPase bands were present in the lower part of the gel, which had no immune-reactivity against F_1 α/β subunits. Extracts obtained by method A also showed the presence of a faint second band just below the ecto-F_0F_1 band with ATPase activity and immune-reactivity against F_1 α/β subunits (Fig. 1a, b), suggesting that a partial degradation of the ecto-F_0F_1 complexes may have occurred during the long centrifugation process. It was concluded that method B was the method of choice for preparing the ecto-F_0F_1 complexes.

Consistent with *hr* CN PAGE results, denaturing electrophoresis followed by immunoblotting confirmed a higher content of F_1 β subunits in PMB than in PMA, in spite of a similar content of flotillin, a lipid-raft marker (Fig. 1c). By comparing the levels of F_1 β subunits on the left and right hand panes in Fig. 1c, we calculate the levels of ecto-F_1 in PMA and PMB to be about 3 and 6 % of the levels observed in mitochondrial membranes, respectively. These values are consistent with those already reported in (Giorgio et al. 2010). In this latter study we also established that mitochondrial contamination of PMA, if any, was very low (less than 1 %). In accordance, Fig. 1d shows that neither PMA and PMB contain the outer mitochondrial membrane protein TOM 20 (Mitochondrial import receptor subunit TOM20). Complex II of the respiratory chain is also absent. Although a proteomic study has detected complex II in lipid-raft preparations from other tissues (Kim et al. 2006), its presence on cell surface has not been confirmed by confocal microscopy of osteosarcoma cells (Yonally and Capaldi 2006). In contrast, and as found in previous reports (De Pinto et al. 2010), both preparations contain the voltage-dependent anion-selective channel (VDAC) of the outer mitochondrial membrane. Hence we conclude, based on the Western blotting results, that neither PMA and PMB are contaminated by mitochondria.

To further exclude that the F_0F_1 complexes detected in plasma membranes do not reflect a mitochondrial contamination, the dose and temperature dependency of digitonin extraction from mitochondria and PMB was compared, since such dependency can be related to lipid composition which is quite different in mitochondria and plasma membrane (Mazzone et al. 2006; Hovius et al. 1990). As evidenced by in-gel ATPase staining of *hr* CN PAGE (Fig. 2), ecto-F_0F_1 complexes can be extracted only at 37 °C, which is consistent with their localization in lipid rafts characterized by detergent-insolubility at low temperature (Kim et al. 2004). Moreover, only the monomeric form of F_0F_1 is visible both at digitonin/protein ratios of 1 g/g and 2 g/g, with its extraction efficiency markedly dependent on digitonin concentration. Increasing the digitonin/protein ratio to 4 g/g does not

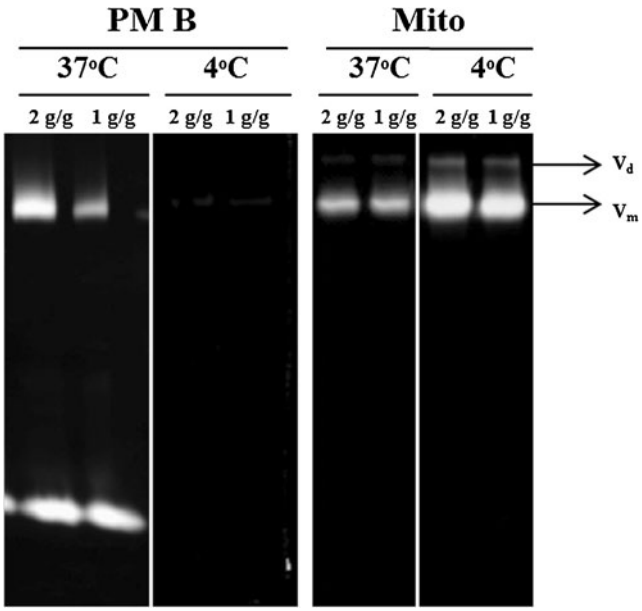

Fig. 2 Detergent and temperature sensitivity of F_0F_1 complexes from mitochondria and plasma membranes. Mitochondrial and plasma membrane proteins (500 μg) were extracted using 1 g/g and 2 g/g of digitonin at 4 °C and 37 °C as described in Methods. Detergent extracts (5 μl for mitochondria and 20 μl for plasma membranes) were separated by *hr* CN PAGE and subsequently stained by in-gel ATPase activity staining

significantly improve ecto-F_0F_1 extraction (data not shown). Conversely, in mitochondria, extraction is optimal at 4 °C, with little dependence on digitonin concentration (Fig. 2). The F_0F_1 dimer is clearly visible in the mitochondrial extracts, as previously reported (Wittig and Schägger 2008; Bisetto et al. 2007). In contrast to the plasma membrane extraction, the efficiency of F_0F_1 extraction from mitochondria is much less at 37 °C than at 4 °C, although both monomers and dimers are still detectable in the extracts. Thus, the different extraction behavior of PMB and mitochondria, i.e. temperature and digitonin concentration dependency, makes unlikely that the ecto-F_0F_1 complexes extracted from plasma membranes at 37 °C arise from mitochondrial contamination, in accordance with the results of Fig. 1d.

Basolateral and apical membranes were then prepared starting from PMB, according to (Cefaratti et al. 2000). The enrichment of both membranes were checked by denaturing electrophoresis followed by immunoblotting for respective markers, i.e. BSEP (bile salt export pump) for the apical and NTCP (sodium taurocholate pump) for the basolateral pole. Figure 3a and c show a clear separation in markers between the separated membrane subfractions, indicating that the apical membranes, had indeed been separated from the basolateral membranes which account for approximately 90 % of the surface area of the hepatocyte plasma membranes (Cefaratti et al. 2000). Immunoblotting also shows that β subunit remains associated to basolateral membranes, whose content is similar to that of PMB. Conversely, β subunit content of apical membranes markedly decreases. All preparations were then subjected to detergent extraction and *hr* CN PAGE followed by in-gel ATPase activity staining. Figure 3b shows that PMB and basolateral membranes contain F_0F_1 complexes having the same molecular mass, but whose activity is higher in the basolateral as compared to the PMB preparation, suggesting that during basolateral separation a partial release of inhibitor(s) occurred resulting in ecto-F_0F_1 activation. Conversely, apical membranes show no detectable ATPase activity (data not shown). Although contamination of non-parenchymal cells can't be excluded, being the starting material a heterogeneous population of cells (Cefaratti et al. 2000), these results strongly suggest that ectopic complexes are solely localized to basolateral pole of hepatocytes, where they can mediate HDL endocytosis.

LC-MS/MS analyses of ecto-F_0F_1 complexes

hr CN PAGE results demonstrated that ecto-F_0F_1 complexes have effectively the same molecular mass of the mitochondrial F_0F_1 monomers (Fig. 1). However, the mitochondrial-encoded subunits a and A6L have low molecular weight, making it difficult to discriminate complete F_0F_1 complexes from those lacking these subunits based on their migration in *hr* CN PAGE. To demonstrate the presence of subunits a and A6L, LC-MS/MS analyses of the *hr* CN PAGE gel bands containing F_0F_1 complexes extracted from mitochondria and PMB were performed. This represents a methodological challenge due the low F_0F_1 content in PM. A6L was identified with a good sequence coverage in the bands obtained from mitochondrial membranes, along with the F_1 subunits α, β, γ, δ and the F_0 subunits b, d and OSCP (Table 1 and Suppl. Table 1). The presence of subunit ε and a was indicated by only one MS/MS spectrum with a statistically significant score for each protein. In the samples of plasma membranes, besides the majority of the F_1 subunits (α, β, γ, δ) and the F_0 proteins b, d and OSCP, subunit A6L was also identified with a sequence coverage of 46.3 % (Table 2 and Suppl. Table 2), which is a value identical to that determined for this protein in the mitochondrial sample. Conversely, subunit a was not identified, possibly because the 85 % of the sequence of this membrane protein is covered by large and quite hydrophobic tryptic peptides that are challenging to be analysed by LC-MS/MS. Since the subunit A6L, like subunit a, is made within the mitochondrion, this finding supports the hypothesis that the ectopic complexes are first assembled in mitochondria and then shuttled to cell surface.

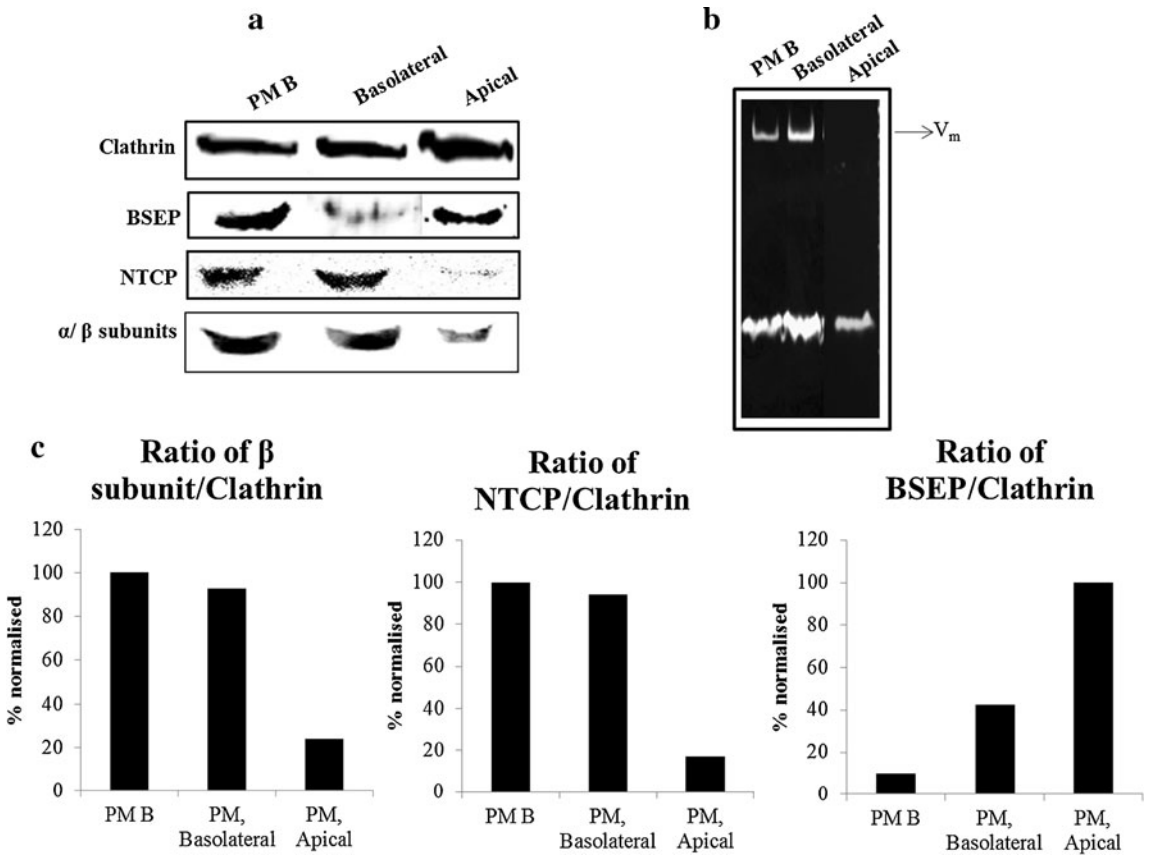

Fig. 3 Ecto-F_0F_1 content of basolateral membranes. Basolateral and apical membranes were prepared from PMB as described in Methods. **a** 40 μg of membrane proteins were subjected to 12 % SDS PAGE and immunoblotted against NTCP (basolateral marker), BSEP (apical marker), β subunit and clathrin. **b** In-gel ATPase staining of PMB, basolateral and apical membranes after the digitonin extraction and *hr* CN PAGE run as in Fig. 1a. **c** histograms representing the ratio of β subunit and markers to clathrin, taken as 100 % the ratios found in PM B

Interestingly, MS/MS analyses revealed the presence of the adenine nucleotide carrier (ADT2) both in mitochondrial and plasma membrane F_0F_1 complexes, suggesting the maintenance of its role in both cellular compartments (Tables 1 and 2). The other proteins identified associated with the F_0F_1 band were quite different for the mitochondrial and plasma membrane samples (Suppl. Tables 3 and 4, respectively), consistent with their different subcellular origin. In particular, there were no detectable electron transfer complexes in the band derived from the plasma membrane samples. There were, instead, the few enzymes of mitochondrial origin involved in cholesterol and lipid metabolism, suggesting a functional relation to the ecto-F_0F_1 complexes which are involved in HDL-endocytosis (Martinez et al. 2003; Malaval et al. 2009).

Intriguingly, mass analyses revealed the presence of the AAA ATPase TERA (Transitional endoplasmic reticulum ATPase) in the band containing the ecto-F_0F_1 complexes from plasma membranes (Table 2 and Suppl. Table 2). The good sequence coverage of the identification and the number of identified peptides suggest that this protein is present in significant quantities. This was further confirmed by immunodetection of TERA in the ecto-F_0F_1 band separated by *hr* CN PAGE from PMB (Fig. 4a). TERA was also clearly detected in basolateral membranes, while was not found in mitochondrial samples (data not shown). TERA is a homohexameric complex involved in a myriad of cellular function including ER-associated protein degradation, and has several alternative localizations including the plasma membrane (Wang et al. 2011). The co-migration of TERA and ecto-F_0F_1 in *hr* CN PAGE could be due to their similar molecular mass, but could also suggest a structural association of the two complexes in plasma membranes. However, when the ecto-F_0F_1 complex was subjected to immunoprecipitation using antibodies against the whole complex, no co-precipitation of TERA was observed (data not shown), suggesting that the two complexes are not stably associated.

To eliminate the possibility that the ATPase activity of TERA might interfere with the in-gel ATPase activity staining of the ecto-F_0F_1 complex, the sensitivity of the activity to NEM, which selectively inhibits the enzymatic activity of

Table 2 List of the proteins identified by LC-MS/MS[a] in the *hr* CN PAGE gel bands containing the F_0F_1 complexes extracted from plasma membranes prepared by method B

Sector	Subunit	Mass (Da)[b]	UniProtKB AC	Coverage (%)[c]	MS/MS peptides[d]
F_1	α	59,754	P15999	46.3	25
	β	56,354	P10719	43.9	18
	γ	30,191	P35435	36.6	10
	δ	17,595	P35434	17.3	3
F_0	b	28,869	P19511	41.4	10
	OSCP	23,398	Q06647	42.7	9
	d	18,763	P31399	47.8	7
	ATP8/A6L	7,642	P11608	46.3	3
Carrier	ADT2	32,901	Q09073	37.2	11
	TERA	89,349	P46462	37.0	26

[a] The identified proteins with their accession numbers and molecular masses are listed. Protein identification was performed with the MASCOT software searching LC-MS/MS data against the sequences *Rattus norvegicus* of the Swiss-Prot database

[b] Molecular mass of the UniProt sequence in the absence of molecule processing

[c] Sequence coverage calculated on the basis of the peptides sequenced by LC-MS/MS

[d] Peptides sequenced by LC-MS/MS. The peptides identified for each protein with their expectation values are listed in Table S2 of the Supplementary Material

TERA (Zhang et al. 1994), was tested. Figure 4 shows that treatment of PM with NEM does not affect the activity of the ecto-F_0F_1 complexes extracted from PM, suggesting that TERA was not responsible for the ATPase activity detected on *hr* CN PAGE. Conversely, incubation of the gel with oligomycin did inhibit the ATPase activity of the ecto-F_0F_1 complexes. This result validates *hr* CN PAGE as tool to detect inhibitor effects (Wittig et al. 2007) and demonstrates that the ecto-F_0F_1 complexes preserve their sensitivity to oligomycin (like the mitochondrial complexes) (Zerbetto et al. 1997). Moreover, this finding confirms the presence of the OSCP subunit (as detected by MS/MS in the ecto-F_0F_1 complexes, see Table 2) and demonstrates the presence of the second mitochondrial-encoded subunit a, which contains the oligomycin binding site (Devenish et al. 2000).

Discussion

This work demonstrates the presence of complete and active F_0F_1 complexes in plasma membranes isolated from rat liver, at about 5 % (g/g protein) of the level observed in mitochondria, in accordance with our previous study (Giorgio et al. 2010). These complexes have been found using two markedly different preparative methods, of which isolation of plasma membranes by isopycnic centrifugation using 2 M sucrose (method B) gives the best results in term of recovery and specific activity of the ecto-F_0F_1 complexes. Moreover, using digitonin extraction, *hr* CN PAGE, in-gel ATPase activity staining and native immunoblotting, we show that ecto-F_0F_1 complexes have a similar molecular mass to the mitochondrial ATP synthase monomers and preserve their sensitivity to the inhibitor oligomycin. Detection of only monomers in PM may be a consequence of the low concentration of ecto-F_0F_1 complexes unable to form polymers, but it is consistent with the uniquely mitochondrial role of ATP synthase dimers to help formation of cristae (Habersetzer et al. 2013), which are absent in lipid rafts (Gorai et al. 2012).

Two lines of evidence exclude the possibility that the ecto-F_0F_1 complexes separated by *hr* CN PAGE were due to mitochondrial contamination, i.e. i) the undetectable mitochondrial contamination of plasma membrane preparations by immunodetection of other mitochondrial proteins; and ii) the different dose and temperature dependencies of the plasma membrane and mitochondrial F_0F_1 complexes to digitonin extraction. The best temperature for ecto-F_0F_1 extraction was 37 °C, while for the mitochondrial complexes it was 4 °C. Such difference is consistent with the localization of the ectopic enzyme in lipid rafts, which require a high temperature for solubilisation of intrinsic proteins (Kim et al. 2004). Moreover, extraction efficiency was markedly dependent on digitonin concentration only in plasma membranes.

The similar molecular mass of ecto-F_0F_1 and mitochondrial F_0F_1 complexes favour their possessing the same subunit composition, raising the question whether whole complexes are routed to cell surface, or whether isolated subunits are assembled there. MS/MS and kinetic analyses of ecto-F_0F_1 complexes allowed us to demonstrate that they contain the mitochondrial-encoded subunit A6L and a, respectively. Since it is highly unlikely that these individual subunits lacking any targeting sequence (Walker 1986) are targeted from the mitochondria to the cell surface, their detection in the ectopic complex makes it unlikely that the enzyme is assembled on plasma membranes. Moreover, in mammalian cell unassembled subunit a is degraded to balance its expression with that of the nuclear ATP synthase gene products, thereby preventing proton leakage across the inner membrane (Rak et al. 2011b). In accordance with this view, no plasma membrane-targeting sequence (or leader peptide) has been identified in ATP synthase nuclear-encoded subunits, and the presence of mitochondrial transit peptide seems to be mandatory for the ectopic localisation of the recombinant β chain in HepG2 (Ma et al. 2010), suggesting that these subunits cannot be routed directly to the plasma membrane. Moreover, ATP synthase assembly factors have not been found in plasma membrane (Vantourout et al. 2010). These findings suggest that the enzyme complex is assembled in mitochondria and that some of the mitochondrial complexes are then routed to cell surface.

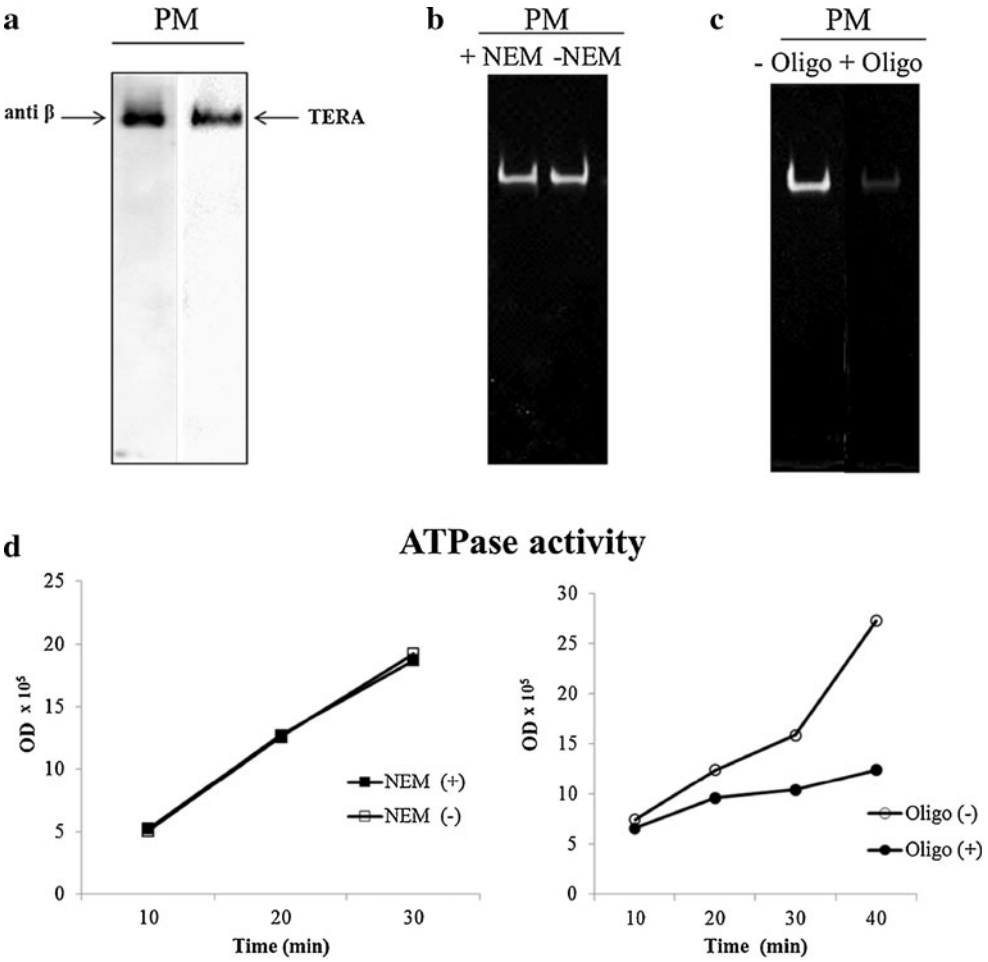

Fig. 4 TERA immunodetection after hr CN PAGE of plasma membrane extracts and NEM and oligomycin sensitivity of ATPase activity staining. **a** Plasma membrane proteins (500 μg) were solubilized using 2 g/g digitonin and were subjected to *hr* CN PAGE and native immunoblotting with mAb against F_1 β and polyAb against TERA. **b** Plasma membrane extract was incubated or not with 1 mM NEM 10 min at 37 °C, separated by *hr* CN PAGE and used for in-gel activity staining. **c** Plasma membrane extract separated by *hr* CN PAGE was preincubated with or without oligomycin before in-gel activity staining as in (Wittig et al. 2007). At time interval, gel was quantified by densitometry. Images in B. and C. refer to 40 min staining. **d** Activity stain OD obtained over time. Slopes of the lines represent the ATPase activities in absence (*white square*) or presence (*black square*) of NEM and in absence (*white circle*) or presence of oligomycin (*black circle*)

This transit mechanism, and its regulation, are still unknown, although different patho-physiological conditions are associated with changes in the ectopic expression of ATP synthase (Giorgio et al. 2010; Zhang et al. 2008; Lyly et al. 2008). An interesting hypothesis is that F_0F_1 complexes may reach the cell surface via vesicular transport, consistent with the recent finding that in synaptic membranes F_1 α chain is in part N-glycosylated, suggesting its transit through the Golgi apparatus (Schmidt et al. 2007). Moreover, considering that distinct mitochondrial-derived vesicles have been characterized, such a mechanism could explain the cell surface location of other selected inner mitochondrial membrane proteins, e.g. three of four respiratory complexes (Yonally and Capaldi 2006) and the adenine nucleotide transporter [this paper]. The presence of this latter is interesting because it suggests the involvement of the mitochondrial transporter in nucleotide delivery to ecto-F_0F_1 across the plasma membrane, where various nucleotide transporters characterized by specific functions have been discovered (Eltzschig et al. 2008).

In conclusion our data demonstrate the presence of complete and active ecto-F_0F_1 complexes on liver plasma membranes. Their distribution between the basolateral and apical surface of hepatocytes has not yet been established, probably due to the difficulty of their direct histochemical observation in tissue sections (Champagne et al. 2006; Vantourout et al. 2008). The selective presence on the basolateral pole of hepatocytes shown in this work is consistent with the role of ecto-F_0F_1 complexes in mediating HDL endocytosis by ADP-dependent activation of the P2Y13 receptor (Martinez et al. 2003).

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